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DOI: https://doi.org/10.1530/REP-17-0425

Reproduction Advance Publication first posted on 18 December 2017 as Manuscript REP-17-0425

Natural and Environmental Oestrogens Induce TGFB1 Synthesis in Oviduct Cells

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Running Title: *Environmental Estrogens Induce TGFB1*

Key Words: Fertilization, endocrine disruptors, infertility, hormones, fallopian tube.

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ABSTRACT

Autocrine/paracrine factors generated in response to 17β-oestradiol (E2), within the oviduct, facilitate early embryo development for implantation. Since transforming growth factor beta1 (TGFB1) plays a key role in embryo-implantation, regulation of its synthesis by E2 may be of biological/pathophysiological relevance. Here we investigated whether oviduct cells synthesize TGFB1 and whether E2 and environmental-oestrogens (EOE's; xenoestrogens and phytoestrogens) modulate its synthesis. Under basal conditions, bovine oviduct cells (OC's; oviduct-epithelial cells and oviduct-fibroblasts; 1:1 ratio) synthesized TGFB1. E2 concentration-dependently induced TGFB1 levels in OC's and these effects were mimicked by some, but not all EOE's (genistein, biochanin-A and 4-hydroxy-2',4',6'-trichlorobiphenyl, 4-hydroxy-2',4',6'-dichlorobiphenyl), moreover, EOE's enhanced ($P<.05$) the stimulatory effects of E2 on TGFB1 synthesis. The OC's expressed oestrogen receptors alpha and beta and aryl-hydrocarbon, moreover co-treatment with ER antagonist ICI182780 blocked the stimulatory effects of E2 and EOE's on TGFB1 synthesis. Treatment with non-permeable E2-BSA failed to induce TGFB1, thereby ruling out the involvement of membrane ER's. Cycloheximide (protein-synthesis inhibitor) blocked E2-induced TGFB1 synthesis providing evidence for *de novo* synthesis. The stimulatory effects of E2 and EOE's, were inhibited ($P<.05$) by MAPK-inhibitor (PD98059), whereas, intracellular-Ca^{2+} chelator (BAPTA-AM) and adenylyl cyclase-inhibitor (SQ22536) abrogated the effects of E2, but not EOE's, suggesting that post-ER effects of E2 and EOE's involve different pathways. Our results provide the first evidence that in OC's, E2 and EOE's stimulate TGFB1 synthesis via an ER-dependent pathway. Exposure of the oviduct to EOE's may result in continuous/sustained induction of TGFB1 levels in a non-cyclic fashion and may induce deleterious effects on reproduction.

INTRODUCTION

The oviduct plays a critical role in reproduction by providing an optimal microenvironment conducive for fertilization and initial stages of embryo development (Li and Winuthayanon 2017). Both rhythmic contraction and relaxation of oviduct smooth muscle cells and ciliary beats of oviduct epithelial cells are critically involved in transporting embryos and gametes (Li and Winuthayanon 2017). Multiple autocrine-paracrine factors generated within the oviduct regulate its function (Li and Winuthayanon 2017). For example, oviduct cells produce nitric oxide (Rosselli et al.1998), hydrogen sulphide (Ning et al. 2014), prostaglandins (Abdel-Razek et al. 2016), thromboxane (Huang et al. 2004), cyclic AMP (Cometti et al. 2003), leukaemia inhibitory factor (LIF1; Krishnan et al. 2013, Reinhart et al.1999), inflammatory cytokines, microRNA's (Ibrahim et al 2015) and endothelin (Jeoung et al. 2010, Reinhart et al. 2003).

TGFB, a 25-kDa homodimer peptide growth factor plays an important role in regulating growth, differentiation and metabolism in many mammalian cell types. It plays an important role in both adult and embryonic growth and development, inflammation and repair, including angiogenesis and regulation of host resistance mechanisms (Jones et al 2006, Li 2014, Monsivais et al. 2017). TGFB induces both autocrine and paracrine effects (Monsivais et al. 2017) and plays an important role in reproduction / the reproductive system (Jones et al. 2006, Li 2014, Monsivais et al. 2017). TGFB has been detected in the human fallopian tube as well as in the human placenta, the human endometrial and embryonic tissues (Jones et al. 2006, Li 2014, Monsivais et al. 2017, Zhao et al. 1994). In human follicles, both theca and granulosa cells produce TGFB1 and TGFB2 (Monsivais et al. 2017), suggesting that it may be

involved in the regulation of follicular growth and oocyte maturation (Monsivais et al. 2017). TGFB1 also regulates endometrial proliferation and differentiation and is involved in the paracrine regulation of trophoblast-endometrium interaction (Monsivais et al. 2017). Importantly, decreased-expression of TGFB1 in the uterus, during trophoblast invasion, results in failed embryo implantation (Monsivais et al. 2017, Singh et al. 2011). Both TGFB1 and TGFB2 produced at the human foetal-maternal interface plays a major regulatory role in the proliferation and differentiation of the trophoblast (Monsivais et al. 2017). Moreover, it regulates the local maternal immune response and prevents foetus rejection (Jones et al. 2006). Thus, TGFB is postulated to be necessary to maintain pregnancy. However, abnormal increases in TGFB are suggested to be a risk factor for recurrent miscarriages (Ogasawara et al. 2000) and in ectopic pregnancy, linked to tubal disorders (Li et al.2011, Shaw et al. 2010).

Ovarian hormones like E2 regulate oviduct physiology. E2 influences oviduct function and the fertilization process by controlling the synthesis of autocrine/paracrine factors within the oviduct (Li and Winuthayanon 2017). In this context, we have previously shown that E2 induces the synthesis of LIF1 (Reinhart et al. 1999) which plays a key role in implantation. Moreover, we demonstrated that endothelin (ET1), a potent contracting and cell survival factor, is synthesized by bovine oviduct epithelial cells and induces the contraction of bovine oviduct segments (Jeoung et al. 2010, Reinhart et al. 2003).

Biological actions of E2 are largely mediated via nuclear oestrogen receptors (ER alpha and ER beta). However, non-genomic and non-nuclear receptors/mechanisms may also participate in mediating the pathophysiological

112 actions of E2 (Hewitt et al. 2016, Rosselli et al.2000). The endocrine effects of E2
113 can be mimicked as well as blocked by chemicals that are structurally similar to E2,
114 bind to ERs and possess estrogenic activity or modulate E2 metabolism by
115 interacting with the aryl hydrocarbon receptor (AhR; Matthews & Gustafsson 2006,
116 Rosselli et al. 2000, Shanle and Xu 2011). These oestrogen-like chemicals are
117 termed EOE's and are classified into two major categories: phytoestrogens (plant-
118 derived oestrogens) and xenoestrogens (man-made oestrogenic chemicals).
119
120 Increasing evidence suggests that EOE's act as endocrine disruptors and
121 interfere with the reproductive process of humans and other species (Rosselli et al.
122 2000, Shanle and Xu 2011). However, the mechanisms involved remain undefined.
123 The fact that some EOE's induce oestrogenic effects, whereas others act as anti-
124 oestrogenic (Rosselli et al. 2000, Shanle and Xu 2011), has further complicated the
125 issue. Recent findings provide evidence that compared to E2, many EOE's known to
126 act as endocrine disruptors have a very low binding affinity for ER alpha and ER beta
127 (Kuiper et al. 1998, Rosselli et al. 2000, Shanle and Xu 2011). This suggests that
128 EOEs may mediate their effects via alternative ER-independent mechanisms such as
129 modulating E2 metabolism (Matthews & Gustafsson 2006, Rosselli et al. 2000).
130
131 Locally synthesized factors play a decisive role in regulating oviduct function
132 by providing an optimal micro-environment for the transport of gametes, the
133 fertilization process, and the development of an early embryo (Li and Winuthayanon
134 2017). We previously demonstrated that oviduct cells produce/synthesize factors like
135 LIF1 and ET1, and E2 simulates their synthesis. Moreover, EOE's like
136 phytoestrogens and polychlorinated biphenyls (PCBs) mimicked the effects of E2 on
137 LIF1 (Reinhart et al. 1999), whereas contrasting effects were observed on ET1

(Reinhart et al 2003). Whether E2 regulates TGFB1 synthesis in the oviduct and whether EOE's mimic the effects of E2 on TGFB1 synthesis remains unknown.

Accordingly, the aims of the present study were to determine the following: (1) whether bovine oviduct cells synthesize TGFB1; (2) whether TGFB1 synthesis is regulated by E2; (3) whether the effects of E2 are mediated via *de novo* synthesis; (4) whether the effects of E2 and EOE's on TGFB1 synthesis are ER operated; (5) whether EOE's modulate the effects of E2 on TGFB1 synthesis; and (6) whether E2 and EOE's influence TGFB1 synthesis via similar intracellular mechanisms.

MATERIAL AND METHODS

Mixed Cultures: Oviducts of young, cyclic, non-pregnant cows were obtained from the local abattoir and the oviduct cells (OC's; mixed population of epithelial cells and fibroblast, 1:1 ratio) were cultured in Ham's F10 (Sigma, Chemie, Buchs Switzerland), containing 10% foetal calf serum (FCS, Sigma, steroid-free), according to our previously published method (Reinhart et al.1999). Briefly, we used confluent monolayers of OC's cultured for 6–8 days. The mixed cell cultures of oviduct epithelial cells (OEC's) and oviduct fibroblasts (OFCs) were characterized immunohistochemically as previously described (Reinhart et al. 1999). Monoclonal antibodies to epithelial cell cytokeratin, (anti-cytokeratin AE1/AE3; Dako Diagnostics AG, Zug, Switzerland.) and antibodies against fibroblast vimentin, (anti-vimentin VIM 3B4; Dako) were used to identify OEC's and OFC's in culture. Stained cells were visualized using peroxidase, anti-peroxidase staining (Dako). TGFB1 antibodies (mouse monoclonal IgG; 25µg/ml; R&D systems) with anti-mouse horseradish

immunoperoxidase staining was used for immunostaining of TGFB1 in cultured OC's. Staining of OC's without primary antibodies was negative control.

Cells in primary cultures or first passage were used to assess TGFB1 synthesis. Prior to experiments with mixed cultures, preliminary studies in cultured oviduct fibroblasts (>97% purity) and epithelial cells (>90% purity) were conducted to assess TGFB1 production. Under basal conditions, oviduct epithelial cells (OECs), oviduct fibroblasts (OFCs) and OCs (OEC's + OFC's 1:1 ratio) produced TGFB1. In supernatants collected from cultured cells at 72 hours, the TGFB1 levels were 611±95 pg/mg protein in OEC's, 845±47 in OFC's and 709±68 in mixed OC's **(Supplemental figure 1A).** The production of TGFB1 in different oviduct cell preparations was comparable and treatment with E2 stimulated its synthesis by ≅ 3.36±0.5 and 2.85±0.24 fold in OEC's and OFC's, respectively. In OC's, the basal levels at 72 hours ranged between 696±14 to 785±70 pg/mg protein in different oviduct preparations and was induced by 3.2±0.35 fold in response to E2 (200ng/ml). Because autocrine/paracrine factors generated by both OEC's and OFC's may regulate the physiology and biology of the oviduct, we decided to use the mixed OC culture system to analyse the effects of natural and EOE's on TGFB1 synthesis.

Oviduct fibroblast cultures: Bovine OFC's were isolated, characterized and cultured as previously described by us (Reinhart et al.1999). For all assays, we used cells in primary cultures or first passage.

Treatment protocol for TGFB1 synthesis by OC's: Sub-confluent monolayers of bovine OC's were washed with HBSS and fed with Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 (Gibco), supplemented with 1%

charcoal-stripped FCS (Sigma, Buchs, Switzerland). After 3 days, we collected the culture medium and assayed immunoreactive TGFB1. To investigate whether TGFB1 synthesis is time-dependent, we measured the TGFB1 level in conditioned media, collected at 24, 48 and 72 hours from OC cultures. To assess the regulation of TGFB1 synthesis by E2 and EOE's, OC's were treated for 72 hours in DMEM/Ham F12 medium (Sigma), supplemented with 1% steroid-free FCS and containing or lacking E2 (0.2, 2, 20, 100, 200 ng/ml; Sigma); genistein (200 ng/ml; Extrasynthèse, France); biochanin A (200 ng/ml; Extrasynthèse, France); 4: 2,4,6-trichlorobyphenyl (TCB, 200 ng/ml, AccuStandard); 4-hydroxy-2',5'-dichlorobiphenyl (4-OH-DCB, 200 ng/ml, AccuStandard); or 4-hydroxy-2',4',6'-trichlorobiphenyl (4-OH-TCB, 200 ng/ml, AccuStandard). To study whether basal and E2 stimulated increase in TGFB1 is due to *de novo* synthesis, OC's were treated for 72 hours with E2 (200 ng/ml) in the presence or absence of cycloheximide (10μM, a protein synthesis inhibitor). To test whether the effects of E2 and EOE's are mediated via ERs, OC's were treated with E2, genistein, biochanin A, TCB, 4-OH-DCB and 4-OH-TDB (200 ng/ml) in the presence of 1uM - 10uM ICI182720 (ER-antagonist; Tocris, Bristol, UK). The rationale for using 1 and 10uM of ICI was based on the findings that the relative binding affinity of E2 and ICI182780 for ERs are 1 and 0.89, respectively. Moreover, the inhibitory potency (IC50) is 0.29 nM. Since, EOE's have very low affinity for ERs as compared to E2 (Kuiper et al. 1998) , together with the fact that 10 fold higher concentrations of ICI have been shown to completely block the ER activating effects of E2, we elected to use a concentration of 1 and 10 uM for the experiments. Moreover, to analyse the possible role of the membrane ERs, OC's were treated with the same concentration of E2 tagged to BSA, which is membrane impermeable (200 ng/ml of E2 is equivalent to 1180ng/ml E2-BSA, Sigma). Finally, to assess involvement of intracellular mechanisms in mediating the effects of E2 and EOE's on

TGFB1 synthesis, we used BAPTA-AM (a membrane permeable calcium chelator, 1μM, Sigma), SQ22536 (adenylyl cyclase inhibitor, 500 μM, Sigma) and PD98059 (MAPK inhibitor, 20 μM, Tocris Cookson LTD, Bristol, UK). For each experiment, the conditioned medium was collected to analyse TGFB1 levels, whereas, the remaining cells solubilized in 0.2% SDS and the protein levels were measured using BCA-protein assay Kit (Soccochim SA, Lausanne, Switzerland). All experiments were in triplicate using three separate cultures derived from different pools of fresh oviducts.

TGFB1 ELISA: The presence of immune-reactive TGFB1 in conditioned medium (200μl aliquots) of OCs was analysed using an ELISA Kit (Quantikine™, human TGFβ1 immunoassay; R&D Systems, Minneapolis, MN, USA). Briefly, 200 μl of activated samples (500 μl samples treated sequentially for 10 min with 100 μl 1N HCl, and 10 min with 100 μl 1.2N NaOH /0.5M Hepes) were added to a microplate coated with recombinant human TGFB sRII and incubated at RT for 3 hours. Subsequently, the plate was washed several times and 200 μl of polyclonal antibody against TGFB1 conjugated to horseradish peroxidase was added and further incubated at RT for 90 min. After washing the plate, 200 μl of substrate solution containing stabilized hydrogen peroxide and chromogen (tetramethylbenzidine) was added. After incubating at RT for 20 minutes, the reaction was terminated by adding 50 μl of 2N sulfuric acid. The optical density was determined using a microplate reader, set to 450 nm, with a wavelength correction, set to 540 nm. According to the manufacturer's specification, the minimal detectable concentration of TGFB1 was less than 7 pg/ml. TGFB1 concentrations were estimated using a standard curve run under identical conditions. TGFB1 concentrations were normalized against total cell protein and the results presented as percent of control.

Western Blotting: Western blotting was used to further confirm the modulatory effects of E2 and EOE's in OCs. Briefly, OCs treated were treated with E2, genistein, biochanin A and 4-OH-TCB (200 ng/ml) in the presence or absence of 10uM ICI182720 (ER-antagonist). After 72 hours, the cells were lysed using 70ul of cell lysis buffer and combined with trichloroacetic acid (TCA; 10%) precipitated conditioned medium fraction of respective samples. Sample aliquots containing 25ug protein were electrophoretically resolved on a SDS gel under non reduced conditions and separated proteins transferred onto nitrocellulose. After blocking with 5% milk in PBS-Tween (Sigma), to prevent unspecific binding, the membranes were incubated with primary antibody for TGFB1 (TGFB1, mouse monoclonal; Santa Cruz Biotechnology, Inc.; dilution 4ug/ml). The membranes were washed and subsequently treated with the corresponding secondary antibody (goat anti-mouse IgG HRP peroxidase (1:5000 dilution). After washing the bands were determined using Super signal west dura luminol substrate (Pierce) and Hyperfilm ECL films (Amersham). Changes in TGFB1 were normalized to B-actin (Sigma; 1:5000 dilution), using the LI-COR system. Briefly, membranes were incubated for 5 min with Newblot Nitro Stripping buffer and then washed 3x 10 min in PBS. Anti-B-actin mouse, Sigma (1:5000 dilution). IR Dye 800 conjugated Anti-mouse IgG LI-COR Biosciences 1:12500. Changes in protein expression were analysed by measuring optical density using Image J software and changes in protein expression presented as optical density ratio between TGFB1 and B-actin.

To assess whether E2 and EOE's activate MAPK pathway, OCs grown for 24 hours in medium devoid of serum were treated for 15 minutes with E2, genistein, biochanin A and 4-OH-TCB (200 ng/ml) in the presence or absence of 10uM ICI182720 (ER-antagonist). The cells were lysed, resolved electrophoretically, and

probed for phosphorylated and non-phosphorylated MAPK using phosphospecific anti-MAPK mouse monoclonal antibody (phosphor-p44/42 MAPK; Thr202/Tyr204; Cell Signaling) and anti-p44/42 MAPK (ERK1/2) antibody (source rabbit; dilution 1:1000; Cell Signaling).Corresponding HRP linked secondary antibody was used for detection. Changes in protein expression were analysed by measuring optical density using Image J software. Changes in protein expression presented as optical density ratio between phosphorylated MAPK (MAPK-P) and non-phosphorylated MAPK.

Receptor Expression Studies: The expression of ERs and the aryl hydrocarbon receptor (AhR) in OCs was assessed using Western blotting. Briefly, OC lysates were probed with antibodies to ER alpha, ER beta or AhR, respectively. Antibodies for ER alpha (purified antiserum to human ER alpha), ER beta (purified antiserum to human ER beta), and AhR (rabbit polyclonal IgG against human AhR protein which recognized AhR at 96 and 122 kDa) were from Alexis, and diluted 1:1000 in buffer containing 1% milk, PBS, 0.2% Tween-20. The secondary biotinylated antibodies (ImmunoPure goat Anti-Rabbit IgG, peroxidase conjugated, Pierce, Illinois, USA) diluted to 1:25000 in 1% milk, PBS, Tween 20 0.2% buffer, during 1h at RT.

Statistical Analysis: Data are presented as a mean $\pm$ SEM of experiments conducted in triplicates (n=3). Statistical analysis was performed using ANOVA or Fischer's least significant test, as appropriate. A value of $P < 0.05$ was considered statistically significant.

289 **RESULTS**

290 **Effects of E2 on TGFB1 synthesis:** We first established the optimal
291 conditions for TGFB1 synthesis by the OCs (OECs plus OFCs; 1:1 ratio). They were
292 used for all experiments unless specified otherwise. **Figure 1A** depicts OFCs
293 positively stained with vimentin (brown staining marked **a;** negatively stained
294 epithelial cells labelled b) in a representative OC culture. Western blotting of lysates
295 **(Figure 1B)** collected from these cultures provides evidence for the presence of ERs
296 alpha and beta, as well as the aryl hydrocarbon receptor (AhR; **Figure 1B** right
297 panel; **See Supplementary Figure 1B for original un-cut blot). Top panel of**
298 **figure 1C shows the oviduct fibroblasts (middle panel) and epithelial cells (right**
299 **panel) positively stained for TGFB1. The left panel depicts the negative control**
300 **(neg-C).** In cultured OCs, the level of TGFB1 increased in a time-dependent fashion
301 and was 581.8±13.3, 720.4±14.3, and 785.7±70.7 pg/mg protein after 24, 48 and 72
302 hours ($p < 0.05$ vs 0 hour), respectively (**Figure 1C**). Under similar culture conditions,
303 we also observed production of immunoreactive TGFB1 in pure OECs and OFCs
304 (**Supplementary Figure 1A**). To mimic the physiologic conditions within the oviduct,
305 we used OCs to study the influence of test agents on TGFB1 production.
306
307 Treatment of OCs with E2 (0.2, 2, 20, 100, 200 ng/ml) for 3 days increased
308 TGFB1 levels in a concentration-dependent manner **(Figure 2A)**. The lowest
309 concentration of E2 that significantly increased TGFB1 levels was 100 ng/ml. At this
310 concentration, E2 increased TGFB1 levels from 100% to 213 ± 15 % ($p<0.05$
311 compared to vehicle treated control; **Figure 2A**). To assess whether increases in
312 TGFB1 levels was solely due to *de novo* synthesis and not due to a release of stored
313 intracellular proteins, OCs were treated with E2 in presence of cycloheximide, a
314 selective protein synthesis inhibitor. Treatment of OCs with cycloheximide

315 significantly inhibited basal and E2 (200 ng/ml) stimulated synthesis of TGFB1
316 **(Figure 2B),** suggesting that E2 induced TGFB1 production is due to *de novo*
317 synthesis.
318
319 The biological effects of E2 are mediated via ERs alpha and beta (Hewitt et al.
320 2016; Levin et al. 2009), moreover its actions can be modulated by ligands (EOE's or
321 endocrine disruptors) for aryl hydrocarbon (AhR) receptors (Matthews & Gustafsson
322 2006; Shanle and Xu 2011). Western blot analysis of cultured OC lysates provided
323 evidence for the presence of both ERs alpha and beta as well as AhR **(Figure 1B).**
324 To assess whether the stimulatory effects of E2 were ER mediated, we tested its
325 effects in the presence and absence of ICI182780, an ER antagonist which binds
326 with equal affinity to both ER alpha and beta (Kuiper et al. 1998). As shown in **Figure**
327 **3A,** ICI182780 abrogated the stimulatory effects of E2 on TGFB1 production in a
328 concentration-dependent manner, suggesting the actions are ER mediated.
329
330 Recent studies provide evidence that E2 mediates its action via nuclear as
331 well as membrane ER **(Levin 2009).** To assess whether E2 stimulates TGFB1
332 synthesis via membrane ER, we treated OCs with membrane impermeable BSA
333 tagged E2. As shown in **Figure 3B**, equimolar concentrations of E2 tagged to BSA
334 failed to induce TGFB1 synthesis suggesting that E2 mediates its stimulatory actions
335 via nuclear and not membrane-ER.
336
337 **Effects of Phytoestrogens on TGFB1 synthesis:** Similar to E2, genistein (200
338 ng/ml) significantly induced TGFB1 synthesis in OCs ($p<0.05$ vs control cells treated
339 with vehicle). Treatment with genistein (200ng/ml) increased TGFB1 synthesis from
340 100% in controls to 191%±19. Similar to genistein, TGFB1 synthesis was significantly

(p<0.05 vs untreated cells) increased in OCs treated with 200ng/ml biochanin-A, a precursor of genistein (**Figure 4A).** The stimulatory effects of both genistein and biochanin A on TGFB1 synthesis were completely blocked in the presence of ER antagonist ICI182780 **(Figure 4A),** suggesting that their effects were ER mediated.

Effects of Xenoestrogens/PCBs: To evaluate the effects of PCBs on TGFB1 synthesis, we treated OCs with 200 ng/ml of TCB, 4-OH-TCB and 4-OH-DCB. Treatment with 4-OH-TCB, but not TCB and 4-OH-DCB, induced TGFB1 production. The PCB 4-OH-TCB was as potent as E2 and increased TGFB1 production from 100% in control to 275%±25.5 at a concentration of 200 ng/ml (**Figure 4B**). Moreover, the effects of 4-OH-TCB (200 ng/ml) on TGFB1 synthesis were blocked by ICI182780 **(Figure 4B**), suggesting they are ER mediated.

Modulation of TGFB1 synthesis by E2 and EOE confirmed by Western Blotting: Although the ELISA assay is well established to accurately assess TGFB1 levels, western blotting was also performed to rule out non-specific interference. Consistent with our observations with ELISA, treatment of OCs with 200ng/ml E2, 4-OH-TCB, genistein or biochanin A induced TGFB1 expression and this stimulatory effect was abrogated in presence of 10μM ICI182780 **(Figure 5).**

Modulatory effects of EOE plus E2: To investigate whether the effects of E2 on TGFB1 synthesis were influenced in the presence of phytoestrogens and PCBs, we studied the effects of E2 (200 ng/ml) in the presence and absence of 200 ng/ml of genistein, biochanin A, TCB, 4-OH-DCB and 100 ng/ml 4-OH-TCB. As shown in **figure 6A and 6B,** EOE's significantly increased the stimulatory effects of E2 on TGFB1 production.

Role of Ca++, PKA and MAPK on E2 and EOE induced TGFB1 synthesis: The mechanisms mediating the effects of E2 and EOEs on TGFB1 synthesis remains unclear. To study some of these possible pathways, cells in the first passage were treated with BAPTA-AM (membrane-permeable Ca2+ chelator, 1 μM), SQ22536 (adenylyl cyclase inhibitor, 500 μM) and PD98059 (MAPK inhibitor, 20 μM). All the compounds showed a partial but significant ($p<0.05$) inhibition of E2 induced TGFB1 synthesis (**Figure 7**). However, in contrast to E2, the stimulatory effects of phytoestrogens and PCBs were only inhibited by the MAPK inhibitor PD98059, suggesting that their effects are specifically mediated via the MAPK pathway and not via intracellular calcium release or the PKA pathway (**Figure 8**).

Western blotting was performed to confirm the involvement of MAPK phosphorylation in mediating the stimulatory actions of E2 and EOE's on TGFB1 production. As shown in Figure 9, treatment with 200ng/ml of E2, 4-OH-TCB, genistein, or biochanin-A induced phosphorylated MAPK and this effect was blocked in OCs co-treated with 10uM ICI182780 (Figure 9). These findings provide evidence that E2 and EOE's activate MAPK-P via ER dependent mechanism.

To assess whether modulation of E2-induced TGFB1 production by various mechanisms (cyclic AMP, intracellular Ca2+, MAPK) are associated with or independent of ERs, OCs were treated with BAPTA-AM, SQ22.536 or PD98059 in the presence and absence of ICI182780. The attenuating effects of ICI182780 were not further enhanced by BAPTA-AM, SQ22536 and PD98050, suggesting that the stimulatory effects of E2 are mediated via a common pathway involving ER-

dependent activation of intracellular calcium release, adenylyl cyclase and the MAPK pathway (**Supplementary Figure 2A).**

Effects in OFC's: Our results from cultured OFC's (> 90%) were comparable to those obtained in OC's. As shown in **Figure 10**, E2 (200 ng/ml), genistein (200 ng/ml), biochanin A (200 ng/ml), as well as 4-OH-TCB (200 ng/ml), significantly induced ($P<0.05$) TGFB1 levels. These effects were partially (in cells treated with E2) or completely reversed (in cells treated with all other compounds) by the ER antagonist ICI182780 (1 and 10μM; **Figure 10).** We also observed similar effects in oviduct epithelial cells **(Supplementary Figure 2B).**

DISCUSSION

In the present study, we provide evidence that bovine OCs synthesize TGFB1 under basal conditions. E2 stimulates TGFB1 production in OCs via ER and involves ***de novo*** protein synthesis. The stimulatory actions of E2 are mimicked by xenoestrogens (PCBs; TCBs, 4-OH-TCB and 4-OH-DCB) and phytoestrogens (genistein, biochanin-A). Moreover, EOE's enhance the actions of E2 on TGFB1 synthesis in additive fashion. E2 stimulates TGFB1 production in OC's by regulating intracellular calcium, cyclic AMP and MAPK, whereas, EOE's induced TGFB1 solely via MAPK, suggesting that the mechanisms via which natural and EOE's induce TGFB1 are different. Our finding that EOE's modulate local synthesis of TGFB1 within the oviduct suggests that it may play an important role in regulating the biology and physiology of the oviduct associated with early embryo development. More importantly, in contrast to the cyclic effects of E2 on TGFB1 synthesis, continuous

419 exposure to EOE's could induce TGFB1 levels in a non-cyclic fashion and may
420 induce deleterious effects on the reproductive system.
421
422 Cell-cell interaction between oviduct epithelial cells and oviduct fibroblasts
423 plays an important role in maintaining the biological and physiological function of the
424 oviduct by generating multiple autocrine/paracrine factors (Li and Winuthayanon
425 2017, Singh et al. 2011). Hence, we employed OC's, a mixed culture of OFCs and
426 OECs (1:1 ratio), to assess TGFB1 synthesis in the oviduct. Our observation that
427 TGFB1 levels increased in the conditioned medium of OCs in a time-dependent
428 fashion suggests that the oviduct continuously synthesizes TGFB1 under basal
429 conditions.
430
431 Treatment with E2 stimulated TGFB1 production in OCs in a concentration-
432 dependent manner, moreover cycloheximide inhibited basal and E2 stimulated
433 TGFB1 production in OCs. This suggests that basal and E2 stimulated TGFB1
434 production is due to *de novo* synthesis and not simply a release of stored intracellular
435 protein. Moreover, cyclic changes in the levels of E2 may regulate TGFB1 production
436 and importantly, influence the biology and physiology of the oviduct in a timely
437 fashion. TGFB1 plays an important role in embryo implantation, growth and
438 differentiation (Li 2014, Li et al. 2011, Monsivais 2017). In this regard, cyclic changes
439 in active TGFB1 expression occurs during the menstrual cycle (Arici et al. 2003).
440 More importantly, increases in TGFB1 levels in the ovarian follicular fluid, following
441 ovarian stimulation for vitro fertilization correlates with pregnancy (Fried et al. 1998).
442 These observations indicate that E2, together with other cytokines, may stimulate the
443 physiological release of TGFB1 within the oviduct and influence the priming and
444 development of the early embryo for implantation.

445

446 Most biological effects of E2 are mediated via ERs alpha and/or beta (Hewitt
447 et al. 2016). Our observation that pre-treatment with ICI182789, an ER antagonist
448 with equal affinity for ER alpha and beta (Kuiper et al. 1998), abrogated the
449 stimulatory effects of E2 on TGFB1, suggests that the effects of E2 are ER mediated.
450 This contention is further supported by our finding that OCs expressed both ERs
451 alpha and beta. Since biologically active ER has recently been identified in the
452 membrane (Hewitt et al. 2016, Levin et al 2009), we also assessed its role in E2-
453 induced TGFB1 synthesis in OC's. In contrast to E2, equimolar concentrations of E2
454 tagged to BSA, which is membrane impermeable, failed to induce TGFB1 synthesis.
455 This suggests that E2 stimulates TGFB1 production via nuclear, but not membrane
456 ERs.

457

458 Similar to E2, EOE's bind to ERs and possess oestrogenic properties. These
459 characteristics enable EOE's to act as endocrine disrupters and potentially induce
460 pathological affects within the reproductive system (Shanke and Xu 2011, Diamanti-
461 Kandarakis et al. 2009). Our findings provide the first evidence that EOE's induce
462 TGFB1 synthesis in bovine OC's. Phytoestrogens, genistein and biochanin A,
463 significantly induced TGFB1 synthesis in OC's. As compared to genistein, biochanin
464 A was less potent in inducing TGFB1 synthesis. Biochanin A is a precursor of
465 genistein, with a 10,000-fold lower binding affinity than genistein for ERs (relative
466 binding affinity of genistein for ER alpha and beta is 4 and 8, respectively, whereas
467 binding for biochanin A is <0.01 for both ER alpha and ER beta; Kuiper et al. 1998).
468 This indicates that the potency of EOE's to induce TGFB1 depends on their binding
469 affinity to ERs. This notion is supported by our observation that the stimulatory
470 effects of both genistein and biochanin A were completely blocked by the ER

antagonist ICI182780. The fact that genistein and biochanin A are established agonists for ER beta suggests its involvement in mediating the stimulatory effects on TGF-B synthesis (Kuiper et al. 1998). E2 binds to both ER alpha and beta and is more potent than genistein and biochanin-A in stimulating TGFB1 synthesis. This suggests that ER alpha may play a role in stimulating TGFB1 synthesis. Experiments using specific ER alpha and beta agonists and antagonists are required to confirm this contention.

Similar to E2 and phytoestrogens, PCB 4-OH-TCB, but not by TCB and 4-OH-DCB, also induced TGFB1 production. As with phytoestrogens, differences in potency between the PCBs may largely be due to the differences in their binding affinity towards ERs. In fact, 4-OH-TCB is the molecule with the highest affinity (Kuiper et al. 1998). Moreover, the affinity for ER increases in ortho-chlorine substituted molecules and further enhanced in the hydroxylated ones. Thus, 4-OH-DCB has a low affinity for ER (Shanle and Xu 2011) and the non-phenolic but ortho-substituted TCB has very low or no affinity to ER (Shanle and Xu 2011). Our observation that ICI182780 blocked the stimulatory effects of 4-OH-TCB on TGFB1 production suggests that the PCB effects are ER mediated. Since EOE's can act as partial agonists or antagonists via nuclear or membrane ERs/GPER, this may, in part, contribute to the differences in action of natural and EOE's.

To confirm the stimulatory effects of E2 and EOE's on TGFB1 synthesis by OCs were real and not due to non-specific cross reactivity with some other proteins, we confirmed their actions using western blotting. Consistent with the results from ELISA assay, we observed upregulation of TGFB1 protein expression (≈26kDa

protein) by E2 and EOE's. Moreover these effects were blocked by ICI182780 (10uM), reaffirming the notion that the effects are ER mediated.

The above findings suggest that EOE's may induce their deleterious effects in the reproductive system by modulating TGFB1. Indeed, TGFB1 plays an important role in embryo growth and development as well as in the regulation of the local maternal immune response to prevent miscarriage (Li 2014, Singh et al 2011). Importantly, TGFB1 is critical in wound healing and fibrotic scar tissue formation and implicated in tubal abnormalities associated with ectopic pregnancy (Shaw et al. 2010). Hence, we assessed and compared the effects of E2 and the EOE's on TGFB1 synthesis by OFC's. Similar to the effects in OC's, we found that EOE's mimic the stimulatory effects of E2 on TGFB1 synthesis in OFC's. Our findings suggest that EOE's can potentially trigger non-cyclic TGFB1 production locally and contribute to pathological remodelling of fallopian tube associated with adhesions.

Our finding that the phytoestrogens and PCBs enhance the stimulatory effects of E2 on TGFB1 production suggests that exposure to EOE's can result in non-physiological increase of TGFB1 in the oviduct and induce deleterious actions on the reproductive process. Interestingly, 4OH-DCB, which was ineffective in inducing TGFB1 alone, enhanced the effects of E2, suggesting that EOE's with weak oestrogenic activity can also modulate oestrogenic responses in the presence of other endogenous oestrogens. Since cyclic generation of E2 allows events and regulatory feedback to take place at the right moment (Kiyama and Wada-kiyama 2015), presence of EOE's may disrupt this balance. Moreover, in contrast to E2, EOE's may accumulate in fatty tissues and be present in the body for a long time, resulting in constant ER activation and adverse health effects (Shanle and Xu 2011).

Whether non-cyclic, abnormally high levels of TGFB1 may play a role in tubal infertility can only be speculated. Increased incidences of ectopic pregnancies have been observed after recurrent surgery for pelvic adhesions, presence of adenomas, fibrotic scar tissues following wound healing, ovarian hyper-stimulation, and local infections, moreover, these conditions are also associated with an increase in TGFB1 levels (Li et al 2014, Shaw et al. 2010, Tonello & Polli 2007, Xiong et al 2013). For example, Li et al (2011) reported high expression of TGFB1 in occluded fallopian tubes. Since TGFB1 is a master regulator of fibrosis (Meng et al. 2016), it is feasible that increased or abnormal exposure to EOE's may induce TGFB1 levels which could adversely influence oviduct function and biology leading to tubal occlusion and dysfunction. Indeed, exposure to exogenous oestrogens like DES has been associated with unfavourable pregnancy outcomes, including ectopic pregnancy (Palmer et al 2001). Although, multiple factors may contribute to tubal disorders, the autocrine/paracrine role of TGFB1 in response to oestrogens and ER activation may be critical in early embryo priming and transport. It is feasible that continuous presence of abnormally high TGFB1 levels, in response to high E2 or EOE's exposure, triggers implantation like conditions within the oviduct leading to ectopic pregnancies.

E2 activates intracellular calcium, adenylyl cyclase and MAPK via non-genomic mechanisms (Nilsson et al 2001). Since these pathways actively induce TGFB1 formation, they may in part mediate the stimulatory effects of E2 on TGFB1. Indeed, similar to ICI182780, intracellular calcium chelator BAPTA-AM, adenylyl cyclase inhibitor SQ22536 and the MAPK inhibitor PD98059, abrogated the stimulatory effects of E2. Since the inhibitory effects of BAPTA-AM, SQ22536 and

548 PD98059 were not additive, nor did they modulate the effects of ICI182780, suggests
549 that they inhibit via a common mechanism linked to ER. This contention is supported
550 by our finding that ICI12780 blocked the stimulatory effects of E2 and EOEs on
551 MAPK phosphorylation. Interestingly, MAPK inhibitor PD98059, but not Ca2+ and
552 adenylyl cyclase inhibitors, abrogated the stimulatory effects of EOE's
553 (phytoestrogen and PCBs) on TGFB1, suggesting that E2 and EOE's mediate their
554 stimulatory actions on TGFB1 via MAPK activation.
555
556 The ER antagonist blocked the effects of phytoestrogens and PCBs
557 suggesting that they are ER mediated. However, we cannot rule out the participation
558 of other mechanisms. Both phytoestrogens and PCBs are AhR ligands and cross talk
559 between ERs and AhRs influence ER expressions and transcriptional activation.
560 Silencing of AhR lowers/inhibits TGFB1 production (Gramatzki et al. 2009) and the
561 OCs express AhR. Interestingly, PD98056, which blocked the stimulatory effects of
562 EOE's, is also an AhR antagonist (Reiners et al. 1998) and may have blocked the
563 effects of EOE's on TGFB1 via this mechanism. Further in-depth studies are required
564 to test these possibilities.
565
566 Almost every cell in the body produces some form of TGFB and express
567 TGFB receptors, suggesting an important role in preventing disease. Within the
568 female reproductive system, TGFB1 is involved in embryo implantation, growth and
569 differentiation, placental differentiation, endometrium proliferation and differentiation,
570 trophoblast-endometrium interaction during trophoblast invasion into the uterus,
571 cytokine network regulation during pregnancy to maintain a healthy foetus, immune-
572 suppressor to regulate the maternal immune response and avoid miscarriage (Jones
573 et al. 2006, Li 2014). TGFB levels peak during the window of implantation, which

prepares the uterine for embryo implantation by modulating immune responses and localized/controlled apoptosis of endometrial stromal cells and tissue remodelling (Li and Winuthayanon 2017). TGFB regulates angiogenesis and its expression at the embryo–uterine interphase, thought to play a critical role in placenta development (Li 2014; Li and Winuthayanon 2017). Although an increase in TGFB1 in follicular fluid following ovarian stimulation and in-vitro fertilization correlates with pregnancy (Fried & Wramsby 1998), abnormally high TGFB1 levels are associated with miscarriages (Ogasawara et al. 2000).

In conclusion, our findings provide evidence that E2 and EOE's regulate TGFB1 synthesis in oviduct cells. EOE mediated non-physiologic stimulation of TGFB1 synthesis within the oviduct may play an important role in governing the role of the oviduct in the biology and pathophysiology of reproduction.

Declaration of Interest: There is no conflict of interest, which could be perceived as prejudicing the impartiality of the research reported.

Funding: Supported by the Swiss National Sciences Foundation grant 32-55738.98 and 31003A-138067.

Acknowledgements: The data presented in this manuscript was, in part, the PhD dissertation (Diss., Naturwissenschaften ETH Zürich, Nr. 14994, 2003) work submitted by Barbara Cometti to ETH Zurich, Switzerland. http://dx.doi.org/10.3929/ethz-a-004540868.

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FIGURE LEGENDS

Figure 1: **Panel A:** Depicts representative photomicrographs (40X mag) of bovine oviduct cells (mixed cultures of oviduct fibroblasts plus oviduct epithelial cells; primary cultures) with positive immunohistochemically labelling of oviduct fibroblasts with monoclonal antibodies against fibroblast vimentin (anti vimentin VIM 3B4) marked with an arrow (a) and negatively stained epithelial cells (b). **Panel B**: Left panel depicts representative Western Blots depicting the expression of ERs alpha and beta in lysates from bovine oviduct cells. **Right Panel** shows representative Western Blot for expression aryl hydrocarbon (AhR) receptors in lysates from bovine oviduct cells. **Panel C:** Top panel shows representative photomicrograph of oviduct fibroblasts (middle panel) and epithelial cells (right panel) with positive immuno-staining for TGFB1 whereas the left panel depicts the negative control. Lower panel depicts bar graph time dependent synthesis of TGFB1 by confluent monolayers of bovine oviduct cells. TGFB1 levels were measured in conditioned medium collected at 0, 24, 48 and 72 hours of culture. The graph represents mean of three different experiments. Data (mean ± SEM) are expressed as pg/mg protein. (* $p < 0.05$ vs 0h).

Figure 2: **Panel A:** Bar graph showing the concentration-dependent effects of 17B-oestradiol (0.2-200 ng/ml) on the TGFB1 synthesis by cultured oviduct cells. Data (mean ± SEM) represent the mean of three different experiments (n=3, in triplicates). All values were normalized to total protein concentration and the amount of TGFB1 synthesized is express as percent (%) of control. (*$p<0.05$ vs control; Cont). **Panel B:** Bar graph showing the modulatory effects of cyclohexamide (CHX; 10μM on 17B-oestradiol (E2; 200 ng/ml) induced synthesis of TGFB1 by cultured bovine oviduct cells treated for 72 hours. Data (mean ± SEM) represents mean of three different

experiments (n=3, triplicates). The values were normalized to total protein concentration and expressed as pg/mg protein (§ represents significant inhibition, $p < 0.05$ vs control or E2; * $p<0.05$ vs control**).**

Figure 3: **Panel A:** Bar graph showing the concentration-dependent inhibitory effects of ER-antagonist ICI182780 (ICI; 1 and 10μM) on 17β-oestradiol (E2; 200ng/ml) induced TGFB1 synthesis in oviduct cells treated for 72 hrs. Data (mean ± SEM) represents the mean of three different experiments (n=3, in triplicates) and values were normalized to total protein concentration. The amount of TGFB1 synthesized is expressed as percent (%) of control. (* $p<0.05$ vs control, Cont; § $p<0.05$ vs E2 alone or -ICI). **Panel B:** Bar graph comparing the effects of 200ng/ml of 17β-oestradiol (E2) tagged with or without BSA (E2 or E2-BSA), on TGFB1 synthesis by cultured bovine oviduct cells. E2, but not E2-BSA, induced TGFB1 formation. Data (mean ± SEM) represent the mean of three different experiments (n=3, triplicates) and values were normalized to total protein concentration. The amount of TGFB1 synthesized is expressed as percent (%) of control (Con; * $p < 0.05$ vs control).

Figure 4: Bar graph showing the modulatory effects of ICI182780 (1 and10 μM), on: **Panel A:** 17B-oestradiol (E2, 200ng/ml), genistein (200ng/ml), biochanin A (200 ng/ml), and **panel B:** TCB (200ng/ml), 4-OH-TCB (200ng/ml) and 4-OH-DCB (200 ng/ml) stimulated synthesis of TGFB1 by cultured oviduct cells, treated for 72 hours. Data (mean ± SEM) represents the mean of three different experiments (n=3, in triplicates) and values were normalized to total protein concentration. The amount of TGFB1 synthesized is expressed as percent (%) of control. (* $p<0.05$ vs control; § $p<0.05$ vs - ICI182780).

Figure 5: Top panel depicts a representative western blot showing the modulatory effects of effects 17B-oestradiol (E2, 200ng/ml), genistein (200ng/ml), biochanin A (Bio-A; 200 ng/ml), 4-OH-TCB (200ng/ml) on TGFB1synthesis by cultured oviduct cells, treated for 72 hours in presence and absence of 10uM ICI182780. Lower panel shows the changes in optical density from the contrast adjusted blot using image J program. The data depicts change in TGFB1/B-actin ratio from three separate experiments (* p<0.05 vs control; § p<0.05 vs - ICI182780).

Figure 6: **Panel A:** Bar graph showing the modulatory effects of 17β -oestradiol (E2; 200 ng/ml), on Genistein (200 ng/ml), biochanin A (BioA; 200 ng/ml) and **(Panel B)** on E2 (200 ng/ml), TCB (200 ng/ml), 4-OH-TCB (100 ng/ml) and 4-OH-DCB (200 ng/ml) stimulated synthesis of TGFB1 by cultured bovine oviduct cells treated for 72 hours. Data (mean ± SEM) represent the mean of three different experiments (n=3, triplicates) and values were normalized to total protein concentration. The amount of TGFB1 synthesized is expressed as percent (%) of control. (*p<0.05 vs control). * p<0.05 versus control (Con), § p<0.05 versus respective treatment in absence of E2 (-E2).

Figure 7: Bar graph showing the modulatory effects of BAPTA-AM (1 μM), SQ22.536 (500 μM) and PD98059 (20 μM) on 17β-oestradiol (E2; 200 ng/ml) stimulated synthesis of TGFB1 by cultured bovine oviduct cells treated for 72 hours. Data (mean ± SEM) represent the mean of three different experiments (n=3, triplicates) and values were normalized to total protein concentration. The amount of TGFB1

synthesized is expressed as percent (%) of control. (*p<0.05 vs control (C), § p<0.05 vs + E2).

Figure 8: Bar graph showing the modulatory effects of BAPTA-AM (1 μM), SQ22536 (500 μM) and PD98059 (20 μM) on environmental oestrogen's induced TGFB1 synthesis in cultured bovine oviduct cells. Cells were treated for 72 hours with genistein (200ng/ml) or 4OH-TCB (200ng/ml) in the presence or absence of BAPTA-AM, SQ22536 or PD98059. Data (mean ± SEM) represent the mean of three different experiments (n=3, triplicates) and values were normalized to total protein concentration. The amount of TGFB1 synthesized is expressed as percent (%) of control. *p<0.05 versus genistein or 4OH-TCB, respectively, in controls, § p<0.05 versus + Genistein or + 4OH-TCB, respectively, in control.

Figure 9: Top panel depicts a representative western blot, showing the modulatory effects of effects 17B-oestradiol (E2, 200ng/ml), genistein (200ng/ml), biochanin A (200 ng/ml), 4-OH-TCB (200ng/ml) on MAPK activation (MAPK-P) by cultured oviduct cells, stimulated in presence and absence of 10uM ICI182780. Lower panel shows the changes in optical density from contrast adjusted blot using Image J program. The graph depicts change in ratio between phosphorylated and non-phosphorylated MAPK-P/MAPK from three separate experiments. (* p<0.05 vs control; § p<0.05 vs - ICI182780).

Figure 10: **Top Panel:** Bar graph showing the modulatory effects of ICI182780 (1μM and 10μM) on 17β-oestradiol (E2; 200 ng/ml), Genistein (200 ng/ml), biochanin A (Bio-A, 200 ng/ml) and **(Bottom Panel)** on TCB (200 ng/ml), 4-OH-TCB (100 ng/ml)

and 4-OH-DCB (200 ng/ml) stimulated synthesis of TGFB1 by cultured bovine oviduct fibroblasts. Data (mean ± SEM) represents the mean of three different experiments (n=3, triplicates) and values were normalized to total protein concentration. The amount of TGFB1 synthesized is expressed as percent (%) of control. *$p<0.05$ vs control; § $p<0.05$ versus – ICI in respective treatments.

Legends to Supplementary Figures:

Supplementary Figure 1A: Bar graph showing TGFB1 by culured bovine oviduct epithelial cells (OECs), oviduct fibroblasts (OFCs) and oviduct mixed cells (OECs : OFCs 1:1 ratio). TGFB1 levels were measured in conditioned medium collected after 72 hours in culture. The graph represents mean of three different experiments. Data (mean ± SEM) are expressed as pg/mg protein/72 hr.

Supplementary Figure 1B: Representative uncut Western blots showing the expression of ER alpha, ER beta (left panel) and Aryl hydrocarbon receptor (right panel) in cultured OCs. The white arrows indicate the specific bands for the proteins.

Supplementary Figure 2A: Bar graph showing the modulatory effects of BAPTA-AM (1 μM), SQ22.536 (500 μM) and PD98059 (20 μM) in presence of ER antagonist ICI182780 (0.5μM) on 17β-oestradiol (E2; 200 ng/ml) induced synthesis of TGFB1 by cultured bovine oviduct cells. Data (mean ± SEM) represent the mean of three different experiments (n=3, triplicates) and values were normalized to total protein concentration. The amount of TGFB1 synthesized is expressed as percent (%) of control. (*$p<0.05$ vs control (C), § $p<0.05$ vs E2 alone).

Supplementary Figure 2B: Bar graph showing the modulatory effects of ICI182780 (1μM and 10μM) on 17B-oestradiol (E2; 200 ng/ml), Genistein (200 ng/ml), biochanin A (Bio-A, 200 ng/ml) and on TCB (200 ng/ml), 4-OH-TCB (100 ng/ml) and 4-OH-DCB (200 ng/ml) stimulated synthesis of TGFB1 by cultured bovine oviduct epithelial cells. Data (mean ± SEM) represents the mean of three different experiments (n=3, triplicates) and values were normalized to total protein concentration. The amount of

TGFB1 synthesized is expressed as percent (%) of control. *p<0.05 vs control § p<0.05 versus –ICI in respective treatment.

Figure 1

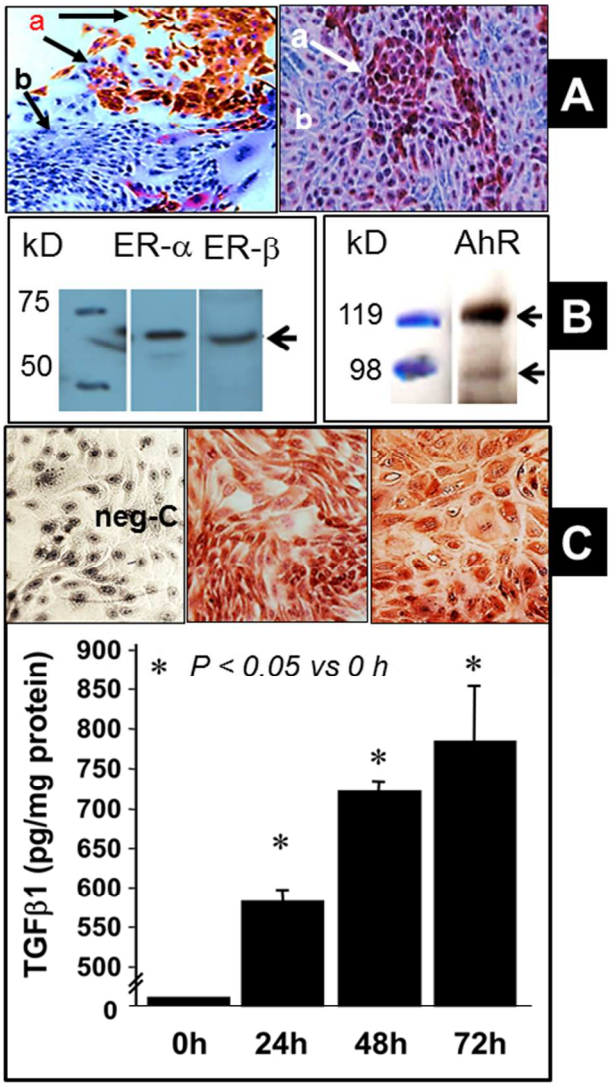

Figure 1

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Figure 2

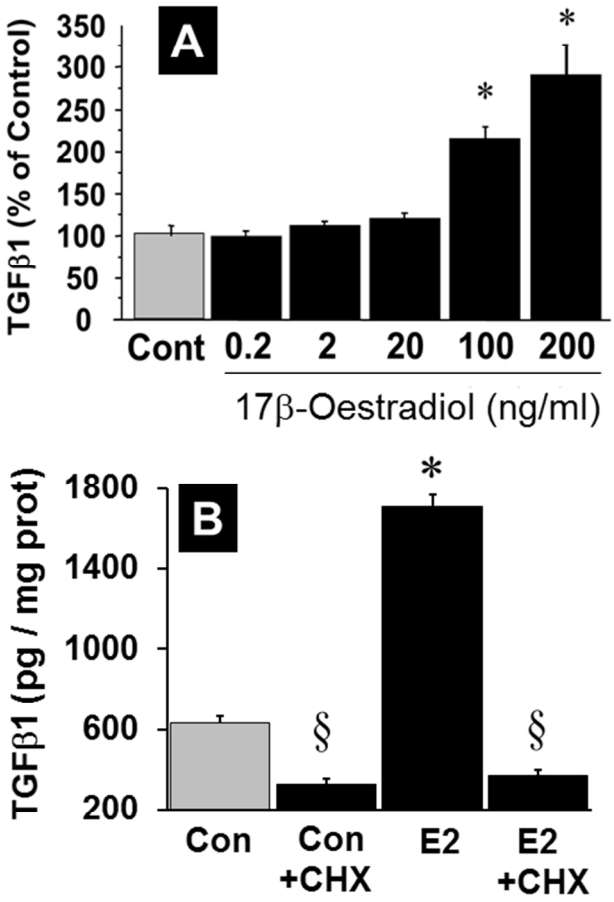

Figure 2

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Figure 3

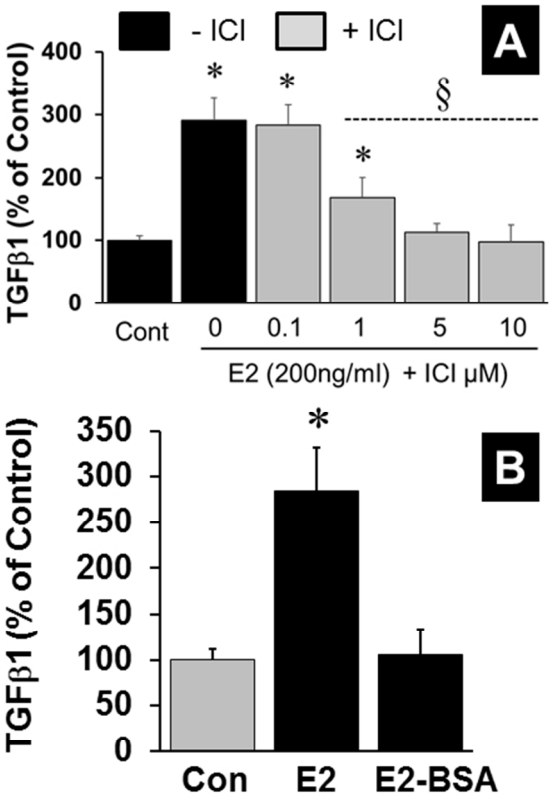

Figure 3

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Figure 4

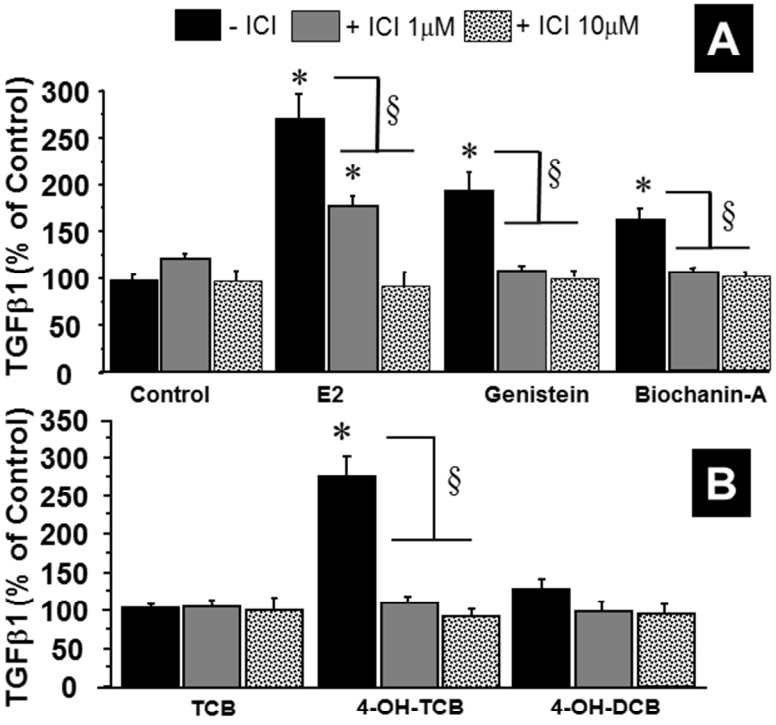

Figure 4

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Figure 5

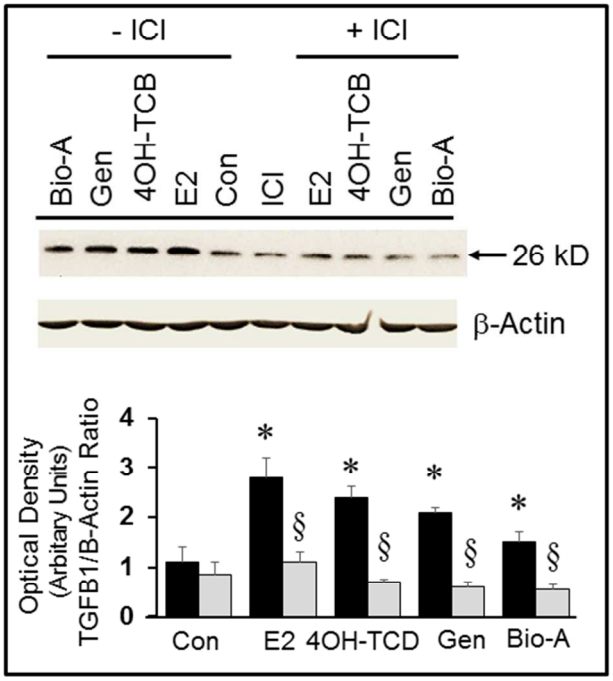

Figure 5

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Figure 6

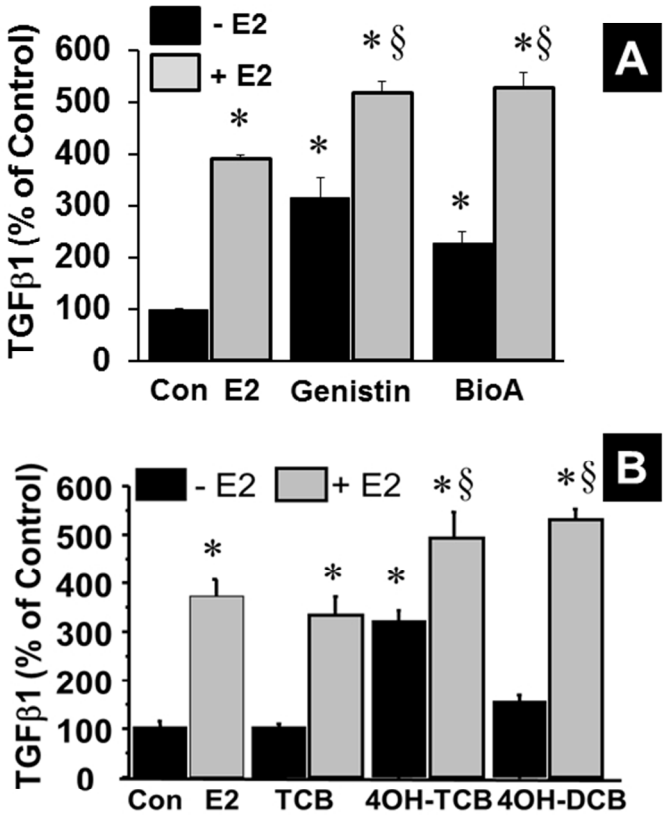

Figure 6

190x254mm (96 x 96 DPI)

Figure 7

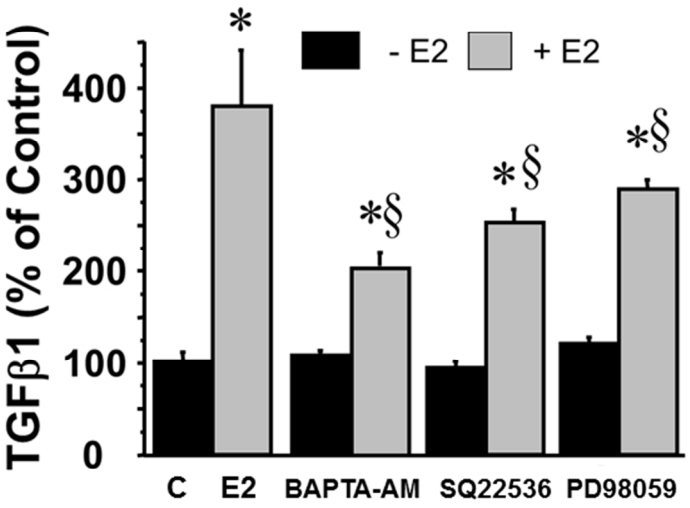

Figure 7

190x254mm (96 x 96 DPI)

Figure 8

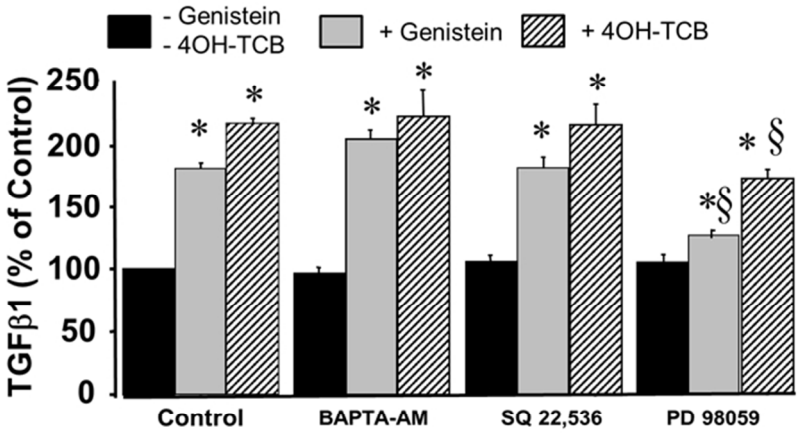

Figure 8

190x254mm (96 x 96 DPI)

Figure 9

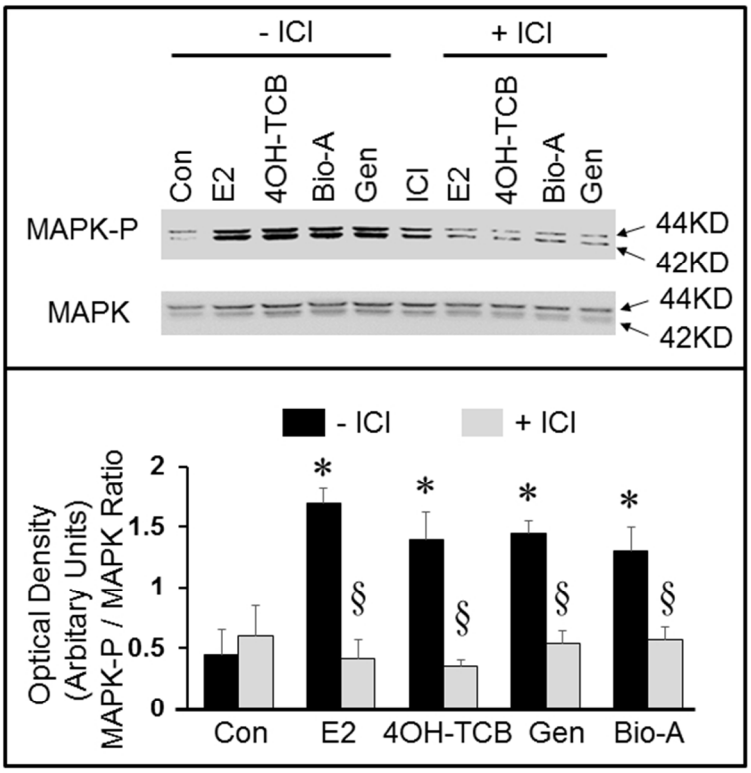

Figure 9

190x254mm (96 x 96 DPI)

Figure 10

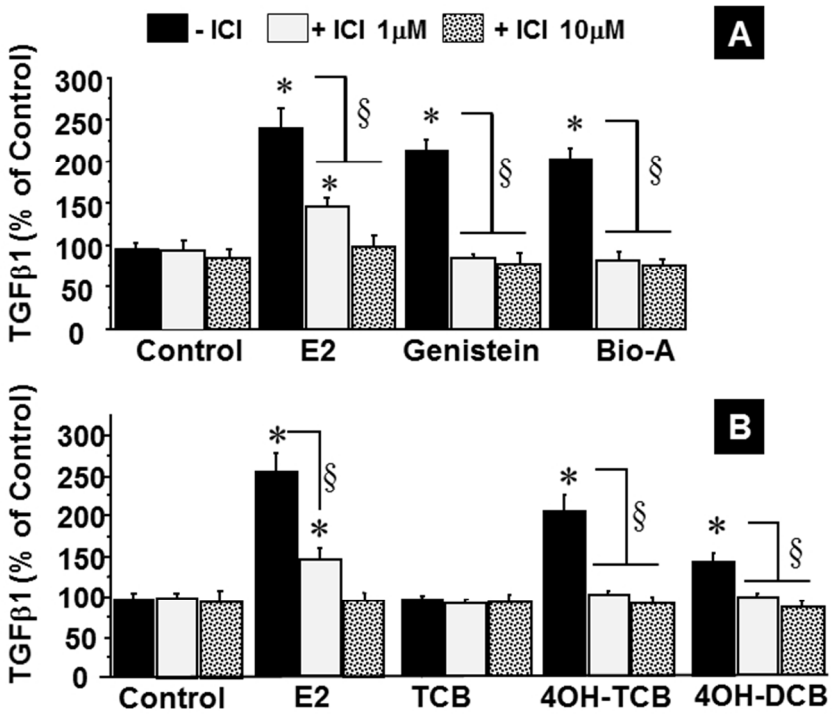

Figure 10

190x254mm (96 x 96 DPI)